glucose-1-C^{14} oxidation would allow for either stimulation in the forward or reverse manner. The direction of the stimulation is then most probably a function of the ratio of the concentrations of oxidized and reduced diphosphopyridine and triphosphopyridine nucleotides.

If in the anterior pituitary ATP synthesis via oxidative phosphorylation can be considered a consequence of DPNH formation, this transhydrogenase activity could provide a sensitive mechanism for its control. ATP thus formed upon stimulation of the transhydrogenase by serotonin or epinephrine could be used in either peptide synthesis or peptide secretion as proposed by Hokin for ACTH.[12]

Summary.—Glucose oxidation in the anterior pituitary is dependent on the concentration of available TPN. There is in anterior pituitary enzymic activity, probably located in mitochondria, which catalyzes the transfer of hydrogen from TPNH or DPNH to 3-acetylpyridine*DPN. This transhydrogenase activity can be stimulated by several compounds of biological importance including serotonin, epinephrine, and estradiol and can be linked to glucose-6-phosphate dehydrogenase. It is suggested that ultimately ATP synthesis may be regulated by this hormonally sensitive pyridine nucleotide transhydrogenase.

The authors are grateful to Helen Fanning, Katalin Piros, and Rose Moquin for their assistance.

* This project was supported by grants from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

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*PROTEIN SYNTHESIS IN ERYTHROID CELLS, I. RETICULOCYTE RIBOSOMES ACTIVE IN STIMULATING AMINO ACID INCORPORATION**

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Communicated by David Rittenberg, October 15, 1962

The synthesis of hemoglobin proceeds in nonnucleated erythrocytes.[1] Studies with reticulocytes indicate specifically that the biosynthesis of globin is initiated on the ribosomes.[2–4]

Tissières and co-workers[5] demonstrated that in *E. coli* nascent protein synthesis

occurs on a particular class of ribosomes ("active" 70 S), which comprises less than 10 per cent of all the ribosomes in cell-free preparations. An RNA characterized by a very high rate of turnover, "messenger" RNA,[6] has been suggested as the intermediate in the transfer of the genetic information for the synthesis of protein. On the basis of subsequent studies with *E. coli*, it was suggested that "messenger" RNA is selectively attached to, or incorporated into, the "active" ribosomes.[7, 8]

In a previous report,[9] data were presented indicating that in reticulocytes capable of protein synthesis there is little or no new formation of the RNA of ribosomes or of a "messenger" RNA. It was of interest to determine if a specific class of ribosomes could be identified as the site of protein synthesis in reticulocytes. In cell-free extracts of reticulocytes, the state of aggregation of the ribosomes can be affected by changes in ion concentration.[10, 11] Recently, Lamfrom and Glowacki,[12] in studying the effect of dissociation on reticulocyte ribosomes, suggested that there are two classes of ribosomes actively participating in protein synthesis, one of which is particularly active.

In the present study, evidence has been obtained that the site of protein synthesis in both intact reticulocytes and a cell-free system prepared from these cells is a class of ribosomes with a sedimentation coefficient of greater than 100 S. No synthesis of ribosomal RNA could be detected in reticulocytes. Taken as a whole, these data indicate that the information for coding amino acids in proteins synthesized by these systems is contained in a relatively stable form in the "active" ribosome particles.

Methods.—Conditions for the incubation of intact reticulocytes with C^{14}-guanine or C^{14}-valine: Reticulocytes were prepared from phenylhydrazine-treated rabbits and washed according to the procedures of Borsook *et al.*[13] The conditions for incubation of the washed cells are given in the legends for the figures. Following incubation, the cells were recovered by centrifugation and washed. The cells were lysed by a short exposure to a hypotonic solution as follows: 1 volume of washed cells was suspended in four volumes of a solution of 1.5×10^{-3} *M* $MgCl_2$ and 10^{-3} *M* Tris chloride buffer, pH 7.5 (Solution I), and agitated for 30 sec. Isotonicity was then rapidly restored by addition of an appropriate volume of 1.5 *M* NaCl. The unlysed cells and cell debris were sedimented at 10,000 rpm for ten min in a Spinco Model L preparative ultracentrifuge, No. 30 rotor. The procedure was carried out at 0°. This procedure preserves the leucocytes and tends to disrupt only the red cells.[14] Lysates obtained in this manner will be referred to as "shock" lysates.

Isolation of RNA: RNA was isolated from washed cells, "shock" lysates, or the sedimented cells remaining unlysed by adding four volumes of a solution of 5 per cent sodium dodecyl sulfate, 10^{-3} *M* Tris chloride buffer, pH 7.5, bentonite, 120 mg/liter and 1.5×10^{-2} *M* naphthaline-1,5-disulphonate. This solution was rapidly mixed with an equal volume of 90 per cent phenol containing 0.1% 8-hydroxyquinoline, and RNA was further purified by the procedure described by Kirby.[15]

Isolation of ribosomes: Ribosomes were isolated from "shock" lysates by the method of Wallace *et al.*[16]

Conditions for incubation of ribosomes in cell-free system: The reaction mixture had a total volume of 1.7 ml and contained: 1 μmole ATP adjusted to pH 7.5 with KOH; 5 μmoles phosphoenol pyruvate; 0.02 mg pyruvate kinase; 0.25 μmole guanosine triphosphate; 1 μmole mercaptoethanol; 5 μmoles $MgCl_2$; 30 μmoles KCl, 0.5 ml of ribosome-free supernatant solution,‡ 0.1 ml of a complete amino acid mixture[13] minus valine; 4×10^{-4} *M* C^{14}-valine (0.3 mc/mM); 50 μmoles Tris chloride buffer, pH 7.5, and 0.25 to 1.0 μg of ribosomes. The mixture was incubated for 15 min at 37°C. Either the reaction was stopped by addition of trichloroacetic acid (TCA) to a final concentration of 5% or, where ribosomes and hemoglobin solution were separated, the

reaction mixture was immediately iced and ribosomes isolated as described above. The ribosome-free supernatant solution containing hemoglobin was dialyzed as described by Dintzis.[4]

Radioactivity incorporated into an acid-insoluble form was determined as described by Allen and Schweet.[17] All samples were counted in a gas-flow counter with a background of 1.8 cpm and were corrected for self-absorption. Ribosome concentrations and RNA were determined by the methods employed by Ts'o and Vinograd.[11]

RNA and ribosomes were characterized by their sedimentation characteristics in sucrose density gradients employing techniques described in detail elsewhere.[9, 18] The conditions of centrifugation are indicated for each type of experiment in the legends for the figures.

The relative concentrations of various sedimenting components in the ribosome preparations were determined on the basis of analysis of a portion of these preparations in the Spinco Model E ultracentrifuge with ultraviolet (UV) absorption optics, at 35,600 rpm and 20° in a 12 mm cell. The measurements were made at a ribosome concentration of 0.7 optical density units (260 mμ) in the buffer solution specified below. The sedimentation coefficients are given in Svedberg units, normalized to 20°C.

Results.—Incorporation of C^{14}-guanine into reticulocyte RNA: The RNA purified from the washed cells incubated with 8-C^{14}-guanine for 3 hr separated into three major peaks after centrifugation in a sucrose density gradient. One sedimented at a relatively slow rate (4 S) and two sedimented more rapidly, corresponding to ribosomal RNA (Fig. 1*a*). The radioactivity in these samples was present in three components corresponding to the peaks of UV-absorbing material. The cells from which this RNA was purified included reticulocytes and leucocytes. The RNA purified from the "shock" lysates of these cells showed a similar distribution of UV-absorbing material, but radioactivity was present in only one major component, that coinciding with the 4 S component (Fig. 1*b*). The C^{14} in the 4 S com-

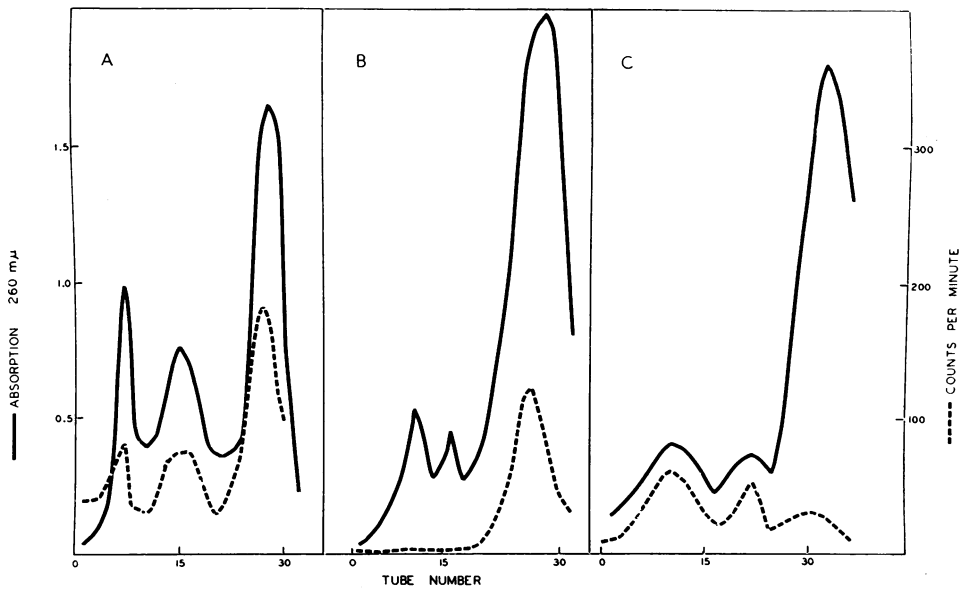

FIG. 1.—Sedimentation of purified RNA labeled with C^{14}-guanine. (*A*): RNA extracted from washed cells as indicated in the text. The conditions of incubation were those employed by Borsook *et al.*,[13] with the addition of inosine, 10 μmole/ml; pencillin, 1 unit/ml, streptomycin, 2 μg/ml, and C^{14}-guanine, 1.5 μc/ml (40 μc/mg). Incubation was for three hr at 37°C. (*B*): RNA extracted from "shock" lysates prepared from an aliquot of the washed cells. (*C*): RNA extracted from cells remaining unlysed after "shock" lysis.

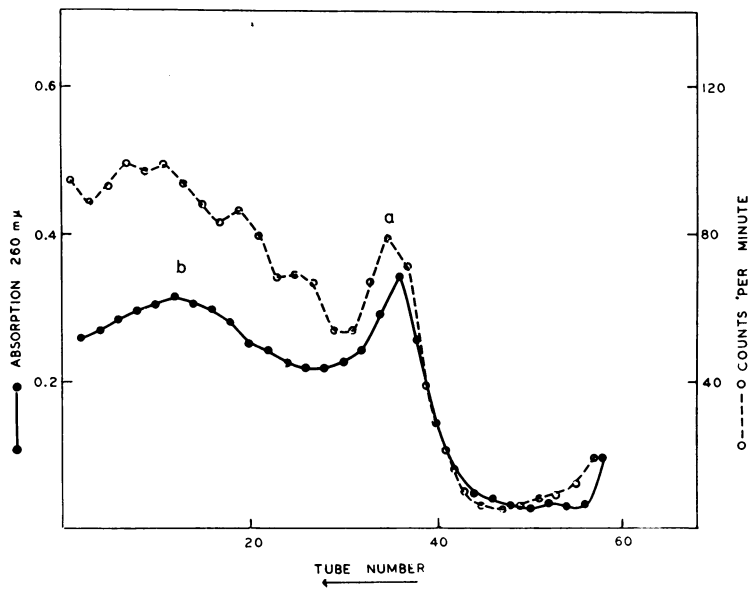

FIG. 2.—Centrifugation of ribosomes in a sucrose density gradient. Ribosomes were isolated from "shock" lysates of cells which had been incubated in the medium employed by Borsook *et al.*[13] with 4×10^{-4} *M* valine (0.3 mc/mM). Incubation was for 15 min at 37°C. The suspension of the ribosomes was dialyzed against 500 volumes of 1.5×10^{-3} *M* $MgCl_2$ and 10^{-3} *M* Tris chloride buffer, pH 7.5 for 3 hr at 0°C, changing the dialysis bath every 45 min. The sucrose density gradient was prepared with the same solution. The conditions of centrifugation for experiments illustrated above and Figures 3 through 5 were 25,000 rpm for 2 hr in Spinco SW-25 rotor.

ponent appears to reflect only counts incorporated into sRNA since the ribosomes isolated from these "shock" lysates had no detectable counts. RNA purified from the sediment of the "shock" lysates which contained leucocytes had radioactivity distributed in the components corresponding to both ribosomal RNA and 4 S material (Fig. 1*c*). The pattern of distribution of radioactivity relative to UV-absorbing material of RNA isolated from each of the three types of preparations as illustrated in Figure 1 was not altered by incubation of cells with C^{14}-guanine for as long as 22 hr. These data suggest that the incorporation of nucleotides into ribosomal RNA observed in total cell preparations reflected primarily the activity of leucocytes. In reticulocytes, the incorporation of nucleotides into ribosomal RNA or any RNA other than the 4 S component proceeds at a slow rate, if at all. The radioactivity in the 4 S component need not reflect a synthesis of RNA. Rather, it could be the result of a conversion of C^{14}-guanine to C^{14}-adenine, which was then incorporated into the terminal adenosine monophosphate of sRNA.[19]

Incorporation of C^{14}-valine into ribosomes of intact cells: The time course of incorporation of C^{14}-valine into soluble protein and ribosomes by intact cells was determined. Employing the conditions indicated in the legend for Figure 2, the rate of incorporation of C^{14}-valine into TCA-insoluble material remains linear over a period of at least 6 to 8 hr, while the level of radioactivity associated with the ribosomal fraction reached a plateau value between 10 and 12 min. These results are similar to those previously reported by Dintzis and co-workers[10] and Rabinovitz and Olson.[3] In subsequent experiments in which the distribution of C^{14}-amino

acid in ribosomes is measured, the reticulocytes were incubated for a period of 15 min or longer as indicated below.

The ribosomes isolated from the "shock" lysates of cells incubated with C^{14}-valine were analyzed by sedimentation in a sucrose density gradient. Ribosomes suspended in a solution of 1.5×10^{-3} *M* $MgCl_2$ and 10^{-3} *M* Tris chloride buffer, pH 7.5, had a pattern of distribution of radioactivity which follows rather closely that of the absorbancy at 260 mμ (Fig. 2). When the ribosomes of similar preparation were dialyzed against a solution containing 1.5×10^{-3} *M* $MgCl_2$, 5×10^{-2} *M* KCl, and 10^{-3} *M* Tris chloride buffer, pH 7.5, and then centrifuged in a sucrose density gradient (Fig. 3), optical density measurements indicate that most of the particles with sedimentation constants of greater than 100 S (Fig. 2*b*) have disappeared and an increase in 78 S particles is evident (Fig. 3*a*). However, the curve of distribution of radioactivity does not follow the same pattern. Rather it is similar to that observed in the absence of KCl. It is evident that the ribosomes with a sedimentation coefficient of greater than 100 S have the highest specific activity (Fig. 3*b*). Analyses of the relative concentrations of particles of different sedimentation coefficient in these preparations of ribosomes were performed in the analytical ultracentrifuge. Ribosomes suspended in 1.5×10^{-3} *M* $MgCl_2$ and 10^{-3} *M* Tris chloride buffer, pH 7.5, contained 30 per cent 78 S, 10 per cent 50 to 60 S, and 60 per cent sedimenting greater than 100 S. The composition of the preparations of ribosomes suspended in 1.5×10^{-3} *M* $MgCl_2$, 5×10^{-2} *M* KCl, and 10^{-3} *M* Tris chloride buffer, pH 7.5, was 70 per cent 78 S, 10 per cent 50 to 60 S, and 20 per cent 100 S or greater particles. These observations indicate that in suspension of ribosomes prepared from rabbit reticulocytes a class of heavy particles, comprising less than 20 per cent of the total ribosomes, is associated with newly formed peptides. These

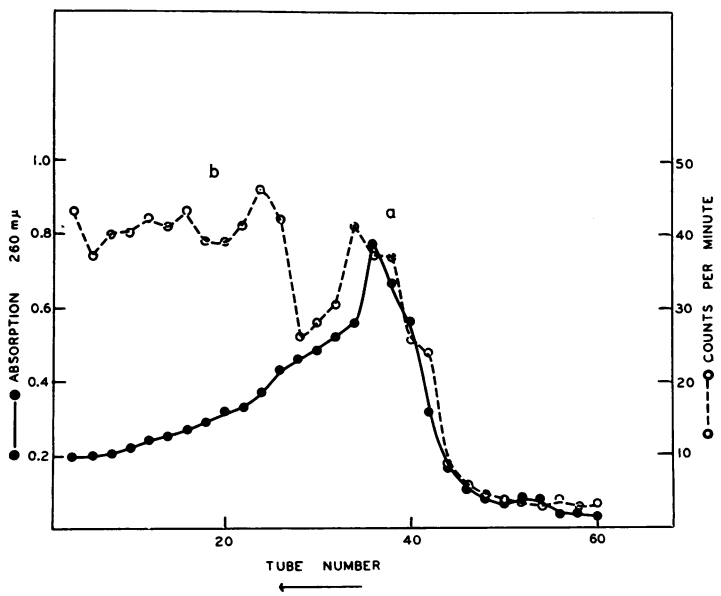

FIG. 3.—Centrifugation of ribosomes in a sucrose density gradient prepared with solutions containing 1.5×10^{-3} *M* $MgCl_2$, 5×10^{-2} *M* KCl, and 10^{-3} *M* Tris chloride buffer, pH 7.5. Conditions of dialysis as in Figure 2 save that the buffer solution was that employed in the preparation of the sucrose solutions.

"active" ribosome particles are relatively resistant to dissociation. Similar observations were made in studies with "active" ribosomes prepared from *E. coli*.[5, 20]

Labeled amino acids associated with the ribosomes could be shown to be subsequently incorporated into soluble protein. This is suggested by studies of the kinetics of the incorporation of C^{14} amino acids which indicate that label initially associated with the ribosomes is incorporated into the hemoglobin of reticulocytes.[3, 10, 17] Additional evidence to support this conclusion was obtained in the following experiments. Labeled ribosomes were prepared from reticulocytes incubated with C^{14}-valine. The ribosomes were then added to the cell-free system described in the *Methods* section, but with unlabeled valine. Following incubation, the ribosomes were reisolated and found to contain no detectable radioactivity. More than 80 per cent of the counts originally associated with the ribosomes were recovered in the soluble proteins.

Incorporation of C^{14}-valine into ribosomes in a cell-free system: Experiments were performed to determine the time course of incorporation of C^{14}-valine into ribosomal particles and soluble protein in the cell-free system. The rate of amino acid incorporation into soluble protein was linear for the initial 10 min, then decreased and halted after 30 to 40 min. Ribosomes became labeled before the soluble proteins and reached a plateau level within 5 to 8 min. These findings are in accord with those of Allen and Schweet.[17] In subsequent experiments, studying C^{14}-valine incorporation into ribosomes in the cell-free system, incubation periods of 6 min or longer were employed as indicated below.

Ribosomes incubated in a cell-free mixture with C^{14}-valine were analyzed by the sucrose density gradient technique (Fig. 4). The fractions of ribosomes corresponding to particles with a sedimentation coefficient in excess of 100 S have specific activities as much as 10-fold greater than those of the 78 S particles. These results indicate that in the cell-free system, as in the intact reticulocyte, ribosomes with a sedimentation coefficient of more than 100 S are particularly active as sites of amino acid incorporation into peptide linkages.

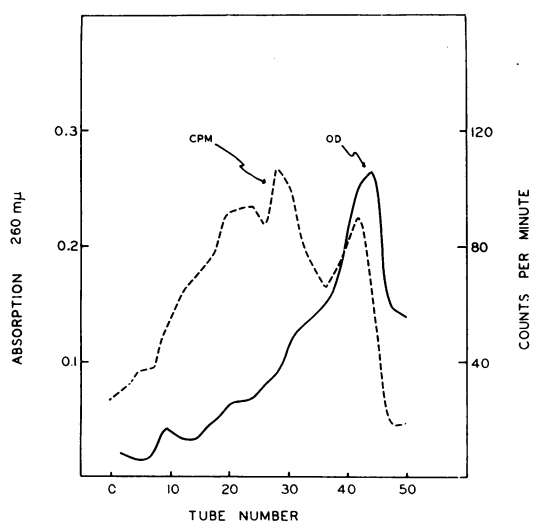

FIG. 4.—Centrifugation of ribosomes in a sucrose density gradient prepared with solutions containing 1.5×10^{-3} *M* MgCl, 5×10^{-2} *M* KCl, and 10^{-3} *M* Tris chloride buffer, pH 7.5. The cell-free incubation mixture was applied to the gradient.

Activity of ribosomes of varying particle size in stimulating amino acid incorporation in cell-free system: Ribosomes isolated from "shock" lysates of reticulocytes were sedimented in a sucrose density gradient, and fractions, corresponding to particles of different size, were separated. These fractions were pooled as indicated in Figure 5. Such ribosome fractions were then assayed for their ability to incorporate C^{14}-valine. The

greatest incorporation (measured as radioactivity made acid-insoluble per mg of ribosomes) was obtained with pooled fractions which corresponded to particles having sedimentation coefficients in excess of 100 S. Little activity was observed with fractions which corresponded to the peak of UV-absorbing material. Certain of the fractions of ribosomes after centrifugation on the sucrose density gradient were also analyzed in the ultracentrifuge to determine the relative concentration of ribosome particles of different sedimentation coefficients. Fraction three (Fig. 5), which had the highest amino acid-incorporating activity per mg of ribosomes, contained 30 per cent 78 S and 70 per cent of ribosomes moving faster than 100 S, while fraction 6, which had a lower amino acid-incorporating activity, contained 80 per cent 78 S and less than 5 per cent of particles moving faster than 100 S. These data indicate that the presence of particles with sedimentation coefficients greater than 100 S is correlated with the ability of the ribosomes to incorporate amino acids.

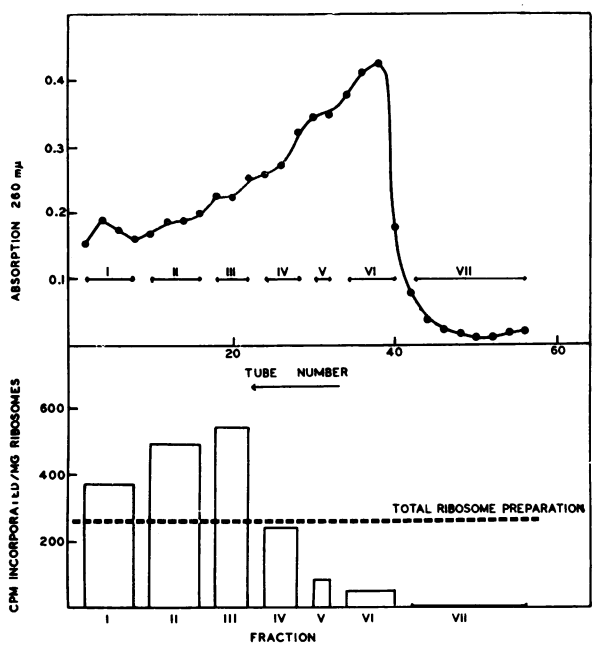

FIG. 5.—Dependence of C^{14}-valine incorporation upon the class of ribosomes. Centrifugation of the ribosomes was performed under the conditions indicated in the legend for Figure 2. Fractions were collected from the sucrose density gradient and pooled as indicated by the Roman numerals in the upper portion of the figure. The ribosome fractions were then added to the cell-free reaction mixture and incubated for 15 min at 37°C. The results are expressed in terms of the cpm in the TCA-precipitable material per mg of ribosomes added.

Discussion and Summary.—These results demonstrate that only a small fraction of the ribosomes prepared from reticulocytes function as sites for protein synthesis. The presence of a special class of ribosomes particularly active in protein synthesis has been shown in studies with *E. coli*[5, 8, 21] and indicated with reticulocytes.[12, 22] The results reported herein indicate that the ribosomes active in protein synthesis are large, with sedimentation coefficients of greater than 100 S. Little or no stimulation of amino acid incorporation was observed by ribosomes of 80 S or smaller.

The present experiments indicate, as has been suggested by previous ones,[9, 23] that reticulocytes, which can synthesize protein for a prolonged period, do not synthesize RNA. In studies to be published, it has been found that reticulocyte ribosome RNA is synthesized prior to the maturation of erythroid cells to the reticulocyte stage.[24] Assuming an average molecular weight of 5×10^5 for the RNA coding for hemoglobin, from data obtained on the rate of hemoglobin synthesis in intact reticulocytes incubated *in vitro*,§ it may be estimated that the order of

magnitude of hemoglobin synthesis per mole of RNA in the "active" ribosomes is 30 moles of hemoglobin per mole RNA per hr. The present observations indicate that the genetic information for protein synthesis in reticulocytes must be incorporated in a relatively stable form in the "active" ribosome particles.

In both *E. coli* and reticulocytes, a heavy ribosome particle, which represents a small fraction of the ribosomes, appears to be the site of new protein synthesis. In contrast to *E. coli*, there is no detectable "messenger" RNA formation in reticulocytes. This suggests that the ability of the heavy ribosome particles to incorporate amino acid may be dependent not on their content of "messenger" RNA alone but on some other factor which is a determinant of the stability of the heavy ribosomes.

Of particular interest are the findings of Reich *et al.*[25] that in mouse fibroblasts concentrations of actinomycin which inhibit RNA synthesis almost completely permitted continued synthesis of protein. Actinomycin, which selectively inhibits DNA-dependent RNA synthesis,[25-27] does not significantly affect the rate of protein synthesis in reticulocytes.[24] Other evidence has been obtained[28, 29] to indicate that no information other than that associated with the ribosomes is necessary for the specific formation of hemoglobin. These observations further support the concept that a stable informational ribosomal complex is a characteristic of protein synthesis in reticulocytes.

The hypothesis of a relatively stable form of RNA which contains the information for protein synthesis may have more general applicability, particularly in mammalian cells which have reached a relatively fixed stage of differentiation. This concept is not incompatible with a role for a turning-over "messenger" RNA which imparts genetic information to ribosomes in cells, such as bacteria or relatively undifferentiated mammalian cells, which have the potential to alter their protein composition in response to environmental changes.

The authors are grateful to Theresa Fiorino for her skillful technical assistance.

* This work was supported in part by grants GM 07368 and CY 2332 of the U.S. Public Health Service.

† Trainee in Hematology under grant 2A-5231 from the U.S. Public Health Service, Department of Medicine, Columbia University.

‡ This supernatant solution was prepared by recovering the upper $^2/_3$ of the supernatant fluid following sedimentation of the ribosomes by centrifugation at 76,000 × *g* for 120 min. This supernatant fluid was again centrifuged at 76,000 × *g* for 120 min, and the upper $^2/_3$ of the fluid was removed and used in the cell-free incubation mixture.

§ This calculation is based on the findings that (*a*) "active" ribosomes represent less than 10 per cent of the total ribosomes and (*b*) 0.8 μmoles of leucine were incorporated into hemoglobin per 10^{10} reticulocytes per hr.[24]

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THE EFFECT OF POLYAMINES AND OF POLY U SIZE ON PHENYLALANINE INCORPORATION*

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Communicated by M. Demerec, October 23, 1962

While investigating an amino acid incorporating system[1-3] from *Salmonella typhimurium* (an enteric organism very closely related to *Escherichia coli*), we found that polyamines exerted a marked stimulatory action upon the formation of protein. The polyamines putrescine and spermidine are the principal cations in these organisms and are known to exist in very high concentrations. Indeed, there are approximately 30 μmoles of polyamine amino groups per gram wet weight of *E. coli*.[4-6] The association of polyamines with nucleic acid has been reported in phage,[5, 7] and in ribosomes.[8-10] Quantitative aspects of this interaction have been studied.[11-15] The present communication deals with the nature of the polyamine stimulation of amino acid incorporation when that incorporation was assayed in the presence of poly U.*

Hershko *et al.*[16] found that polyamines could replace a large part of the Mg^{++} in a mammalian amino acid incorporating system. Mager *et al.*[17] have added polyamines to an *E. coli* incorporating system and in contrast to our results found negligible stimulation. However, in their systems,[16, 17] unlike ours, messenger RNA (e.g., poly U) was limiting (see *Discussion*).

An additional result of this investigation was the finding of an optimal chain